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FOR:	*ALPHA-FETOPROTEIN PEPTIDES AND USES THEREOF*		

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TRANSMITTAL LETTER

Transmitted herewith for filing in the above-referenced patent application are the following documents:

1. Response to Final Office Action dated August 25, 2003 (7 pgs);
2. Declaration of Thomas Andersen under 37, C.F.R. § 1.132 (3 pgs.);
3. Exhibit A (6 pgs.);
4. Courtesy copy of Nomura *et al.* (1991) (5 pgs.); and
5. Return postcard

This Response is being filed on or before the shortened statutory period response date of November 25, 2003. No fee is believed required. However, the Commissioner is authorized to credit any overpayment or charge any deficiencies to Deposit Account No. 50-0311, Reference No. 19705-010. A duplicate copy of this transmittal letter is enclosed.

If the enclosed papers are considered incomplete, the Mail Room is respectfully requested to contact the undersigned collect at (617) 542-6000, Boston, Massachusetts.

Respectfully submitted,

Dated: November 25, 2003

Ivor R. Elrifi, Reg. No. 39,529
Naomi S. Biswas, Reg No. 38,384
Charles E. Bell, Reg. No. 48,128
Attorney for Applicants
c/o MINTZ, LEVIN
One Financial Center
Boston, Massachusetts 02111
Telephone: (617) 542-6000
Facsimile: (617) 542-2241

Please address all correspondence to Customer No.: **30623**.

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HIV-EP2, a New Member of the Gene Family Encoding the Human Immunodeficiency Virus Type 1 Enhancer-binding Protein

COMPARISON WITH HIV-EP1/PRDII-BF1/MBP-1*

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Nobuo Nomura‡, Mu-Jun Zhao§, Takahiro Nagase§, Toshio Maekawa§, Ryotaro Ishizaki¶, Satoshi Tabata‖, and Shunsuke Ishii§**

From the ‡Laboratory of Molecular Biology, Institute of Gerontology, Nippon Medical School, Nakahara-ku, Kanagawa 211, the §Laboratory of Molecular Genetics, Tsukuba Life Science Center, Institute of Physical and Chemical Research (RIKEN), Tsukuba, Ibaraki 305, the ¶Laboratory of Molecular Oncology, Nippon Veterinary and Zootechnical College, Nakahara-ku, Kawasaki, Kanagawa 211, and the ‖Department of Biology, Faculty of Science, Nagoya University, Chigusa-ku, Nagoya, Aichi 464, Japan

At least two different types of proteins, NF-κB/KBF1 and HIV-EP1/PRDII-BF1/MBP-1, which are members of a family of *rel* oncoproteins and metal-finger proteins, respectively, bind to the human immunodeficiency virus type (HIV-1) enhancer. As a new member of a HIV-EP1 family that is expressed at a high level in T cells, we have isolated cDNA clones of HIV-EP2 by cross-hybridization with HIV-EP1 cDNA. HIV-EP2 protein consists of 1,833 amino acids and has a molecular weight of 211,000. HIV-EP2 protein is highly homologous with HIV-EP1/PRDII-BF1/MBP-1 in three regions. These three regions contain the potential nuclear localization signal followed by a Ser/Thr-rich region, the DNA-binding domain consisting of a metal-finger structure, and a cluster of acidic amino acids. The DNA-binding property of HIV-EP2 was similar to that of HIV-EP1. Northern blot analysis of HIV-EP2 mRNA indicated relatively high expression in the T cell line Molt-4 and in some tumor cell lines. Furthermore, like HIV-EP1, expression of HIV-EP2 mRNA was greatly induced by mitogen and phorbol ester treatment of Jurkat T cells, suggesting that HIV-EP2 acts in HIV production from latently infected T cells.

The enhancer of human immunodeficiency virus type 1 (HIV-1)[1] contains two 10-bp repeats that are homologous to the immunoglobulin κ gene enhancer (1). Similar sequences are also found in the enhancer of a class I major histocompatibility (MHC) gene (2) and the β_2-microglobulin gene (3). Multiple proteins that bind to this sequence were identified: H2TF1 found in many types of cells (4), NF-κB consisting of a 50- and 65-kDa polypeptide (5), 48-kDa KBF-1 from mouse BW5147 thymoma cells (6), 51-kDa NF-κB from Namalwa Burkitt lymphoma cells (7), 36–42-kDa polypeptides from human BALL-1 B cells (8), 57–60-kDa EBP-1 from HeLa cells (9), and 86-kDa HIVEN86 found in activated human T cells (10). NF-κB and KBF-1 bind with approximately equal affinity to the site in the MHC enhancer and to a related sequence in the regulatory region of genes such as β_2-microglobulin, but H2TF1 binds with about 10-fold higher affinity to the MHC enhancer site than to the β_2-microglobulin site (3, 11). Differences between H2TF1 and NF-κB were also noted in their contacts with DNA, their inducibility, and cell type distribution (12). NF-κB is present in the cytoplasm in sequestered form that can be activated by phosphorylation of its inhibitor IκB (13, 14). These data indicate that these proteins appear to have overlapping yet distinctive functional properties.

To analyze the structure and function of HIV enhancer-binding protein, we have isolated a cDNA clone encoding HIV-EP1 that specifically recognizes the HIV enhancer (15). Recently, PRDII-BF1 and MBP-1, which were isolated using the interferon β gene promoter probe and MHC enhancer probe, respectively, were shown to be identical to HIV-EP1 (16, 17). This protein has a relative molecular mass of 298 kDa, and contains two zinc fingers in the DNA-binding domain which has the highest affinity for MHC and β_2-microglobulin sites and 5- to 10-fold lower affinity for the HIV-enhancer site. The level of HIV-EP1/PRDII-BF1/MBP-1 mRNA is very low in T cells, but it is induced by treatment of cells with mitogen and phorbol ester (17), suggesting that this protein acts in T cell activation. Recently it was demonstrated by cDNA cloning that the DNA-binding subunits of NF-κB and KBF1 were identical, and they are members of a family of proteins that includes *rel* oncoproteins and *Drosophila dorsal* gene product (18, 19). Therefore, two different types of proteins, the *rel*-related protein and metal-finger protein, can bind to the same site.

To identify the related protein that is expressed at a high level in T cells, and to identify the domain structure required for its function by comparison with the related proteins, we isolated cDNA clones encoding HIV-EP2 by cross-hybridization with the HIV-EP1 probe. We describe here the nucleotide sequence of overlapping cDNA clones which encodes a protein of 1833 amino acids. In addition to the DNA-binding domain containing the zinc-finger structure, two other regions that contain a Ser/Thr-rich sequence and a cluster of acidic amino acids, respectively, are highly conserved between HIV-EP2 and HIV-EP1/PRDII-BF1/MBP-1. A comparison of the levels of HIV-EP2 mRNA expressed in various cells indicates a high expression in the T cell line Molt-4 and some tumor cell lines. Induction of expression of HIV-EP2 mRNA by mitogen

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) M60119.

** To whom correspondence should be addressed.

[1] The abbreviations used are: HIV, human immunodeficiency virus; bp, base pair(s); MHC, major histocompatibility complex; PHA, phytohemagglutinin; PMA, 4β-phorbol 12-myristate 13-acetate.

and phorbol ester treatment of T cells suggests that HIV-EP2 is also a regulatory component of T cell growth control.

EXPERIMENTAL PROCEDURES

Cloning and Sequencing of HIV-EP2 cDNA—Probes used for screening of cDNA libraries correspond to the region of the metal-finger structure of HIV-EP1 and were prepared as follows. Two oligonucleotides, which correspond to nucleotides 121–169 of the sense strand and nucleotides 198–150 of the antisense strand of HIV-EP1 cDNA (nucleotide numbering system is described in Ref. 15), were synthesized. Both oligonucleotides have some mismatches with the HIV-EP1 cDNA at several positions corresponding to the third letter of some amino acids which were not conserved between the metal-finger structures of HIV-EP1 and other reported proteins. Two oligonucleotides were annealed and the complementary sequences filled in using the Klenow fragment with [α-^{32}P]dCTP. Another probe was also prepared in a similar way using two oligonucleotides which corresponded to nucleotides 199–249 of the sense strand and nucleotides 279–230 of the antisense strand of HIV-EP1 cDNA. Two oligonucleotide probes cover the whole region of metal-finger structure of HIV-EP1 (amino acids 41–93). The 270-bp DNA fragment derived from HIV-EP1 cDNA (nucleotides 1–270 in Ref. 15) was also used for screening cDNA libraries. The human cDNA libraries used in this work were generously provided by D. P. Dialynas (a λgt10 cDNA library from mRNA of the T cell line HPB-MLT (20)) and J. E. Sadler (a λgt11 cDNA library from mRNA of endothelial cells from the umbilical vein (21)). Libraries were screened as described (22).

Relevant DNA fragments were isolated from phage clones by digestion with appropriate restriction endonucleases and were cloned into M13mp18 and pUC118 vectors. Sequence analysis was done by the dideoxy chain termination method with modification (23, 24). More than 95% of the nucleotide sequence presented was confirmed either by sequencing the opposite strand or by sequencing the same strand from different initiation sites. The complete nucleotide and deduced amino acid sequences are available from the GenBank sequence data base.

Homology studies and other computer analyses were done with the UWGCG (25) and IDEAS (26) programs in a VAX/VMS computer.

Expression of HIV-EP2 in Escherichia coli—The *lac*Z-HIV-EP2 expression plasmid pAR2106-*lac*Z-HIV-EP2 was constructed using the plasmid pAR2106*lac*Z (27), in which the protein coding region of the *lac*Z gene was linked to the T7 late promoter and a Shine-Dalgarno box, and the cDNA clone λHIV-EP2-4. To generate the plasmid pAR2106*lac*Z-HIV-EP2, the *Eco*RI fragment of the plasmid pAR2106*lac*Z, which contains the COOH-terminal portion of the *lac*Z gene and the pBR322 sequence (nucleotides 1–375 in PBR322 plasmid), was replaced by the 870-bp *Eco*RI fragment of the cDNA insert of λHIV-EP2-4. This 870-bp *Eco*RI fragment encodes the 286 amino acids of HIV-EP2 which contains its DNA-binding domain consisting of two metal-finger structures. In this construct a translational termination codon is generated immediately past the COOH terminus of the portion derived from HIV-EP2 (in the linker region).

Bacteria BL21(DE3) carrying pAR2106*lac*ZHIV-EP2 were grown and the expression of the fusion protein was induced by the addition of isopropyl-β-D-thiogalactopyranoside as described before (28). The insoluble material containing the *lac*Z-HIV-EP2 fusion protein was prepared and solubilized by urea as described (29). The *lac*Z-HIV-EP2 fusion protein was purified using a sequence-specific DNA affinity column in which the synthetic HIV enhancer oligonucleotide was used as a ligand.

In Vitro Transcription and Translation—Using the cDNA inserts of λHIV-EP2-1 and λHIV-EP2-2, the cDNA containing a whole protein-coding region was constructed and cloned into the pSP65 plasmid vector to generate pSP65HIV-EP2. This cDNA was modified by site-directed mutagenesis to generate the Kozak consensus nucleotides required for efficient initiation (30). *In vitro* transcription from the *Bgl*II-digested pSP65HIV-EP2 plasmid DNA template with SP6 RNA polymerase was done as described by Melton *et al.* (31). *In vitro* translation of this RNA using a rabbit reticulocyte lysate (Promega) generated the HIV-EP2 protein containing the NH_2-terminal 1,469 amino acids.

Analysis of DNA-binding Activity—For DNase I footprinting, two complementary oligonucleotides corresponding to nucleotides −115 to −70 of the HIV long terminal repeat were synthesized. After the 5′-end of one strand was labeled, the two strands were annealed and used for DNase I footprinting. This probe contains two 10-bp repeats that are related to the κB site. Binding of the bacterially expressed β-galactosidase-HIV-EP2 fusion protein to a ^{32}P probe, DNase I digestion, and analysis of digestion products were done as described (29). For gel shift assays, three different DNA probes containing the HIV enhancer, the mouse MHC class I enhancer, or the immunoglobulin κ gene enhancer were used. The sequences of these DNAs have been described (8). Rabbit reticulocyte lysate translations of RNA derived from *Bgl*II digests of pSP65-HIV-EP2 were incubated for 30 min at 25 °C with 1 ng of ^{32}P-labeled DNA probe and 1 μg of poly(dI-dC), and the reaction mixture was analyzed by electrophoresis as described (8).

Northern Blot Analysis—Total cellular RNA or cytoplasmic RNA was isolated (32), and poly(A)$^+$ RNA was purified using oligo(dT)-cellulose or oligotex-dT30 (Dai-ichi Kagaku, Co.) as described by the supplier. RNA was fractionated on 0.7% agarose gel, transferred to a filter, and blot hybridized as described (33, 34). Cell lines which were used as a source of RNA were derived from a neuroblastoma (NB-1, TGW-III-nu, NB39-nu), myeloid cells (KG-1), T cells (Jurkat), a T cell lymphoma (Molt-4), a mesothelioma (TC8), an arrhenoblastoma (TC25), thyroid (TC78, TC80), lung (NMS83) colon (Colo320DM), vulva (A431), and breast (MCF-7).

RESULTS

Cloning and Characterization of HIV-EP2 cDNAs—To obtain cDNA clones encoding the HIV-EP1-related protein we screened cDNA libraries using the probes that corresponded to the DNA-binding domain of HIV-EP1 comprising the metal-finger structure under conditions of reduced stringency. Two clones, λHIV-EP2-1 and λHIV-EP2-5, were isolated from a cDNA library prepared from the T cell line HPB-MLT, and four clones, λHIV-EP2-2, λHIV-EP2-3, λHIV-EP2-4, and λHIV-EP2-6, were isolated from a cDNA library prepared from umbilical vein. λHIV-EP2-6 was obtained by rescreening the cDNA libraries with the 1.2-kilobase *Eco*RI-*Hin*dIII fragment of λHIV-EP2-1 as a probe under stringent conditions. Restriction endonuclease analysis demonstrated an identical map in the regions overlapping in those six cDNA fragments (Fig. 1).

To determine the size of the protein encoded by the full length HIV-EP2 cDNA, we determined the complete nucleotide sequence of a set of overlapping clones. The sequence revealed an open reading frame of 5,499 nucleotides. An in-frame termination codon was present 51 bp upstream of the initiator ATG codon, although the flanking nucleotides did not match the consensus sequence of Kozak (30) (data not shown). The nucleotide sequence of the open reading frame was translated into its corresponding 1,833-amino acid sequence (Fig. 2). The molecular weight of the HIV enhancer-

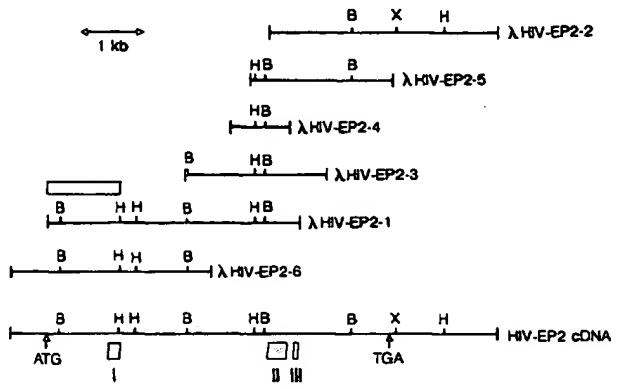

FIG. 1. **Structure of the human HIV-EP2 cDNA clones.** Restriction endonuclease (*B*, *Bam*HI; *H*, *Hin*dIII; *X*, *Xba*I) cleavage map of each cDNA clone is shown. At the bottom is a composite HIV-EP2 cDNA map derived from the above cDNAs. The translation initiation codon, termination codon, and regions I, II, and III (see Fig. 3) are indicated. The probe used for obtaining λHIV-EP2–6 is shown as an *open bar*.

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MLKGISSSSL KEKKLSPGDR VGYDYDVCRK PYKKVEDSET PKQNYRDISC   50
LSSLHHGGEY FMDPVVPLQG VPSHPGTTCE NRKRRKEKSV GDEEDTPHIC  100
SSIVSTPVGI HASDYDPKLQ HQEGVRSGPA HAGHENLSHG HTERFDPCRP  150
QLQPGSPSLV SEESPSAIDS DKHSDLGGRK PPGNVISVIQ HTNSLSRPWS  200
FERSESAELV ACTQDEAPSP SETCDSRISE APVSPEHAPP GDGAESGGKP  250
SPSQQVQQQS YHTQPRLVRQ HNIQVPSIRV TEEPDKPEKE KEAQSKEPEK  300
PVEEPQTPQR SETLSQLPAE KLPPKKKRLR LADCHESSGS SSPESTGTGL  350
SRSPSQESNL SHSSSPSHSP ERRGTSKLSA LPKQDEPGKH SEFLTVPAGS  400
YSLSVPGHEH QREHRRCSSE QSPCPHPAEV PSVRSKSPDY GNLSHAPVSG  450
AAASTVSPSR ERKKCPLVRQ ASPSGSPEIS QGEVGHDQSV KQEQLEHLHA  500
GLRSGDHHGP PAVLPPLQQE DPGKQVAGPC PPLSSGPLHL AQPQIHHEDS  550
QESLRNPLIQ PTSYHTSKHL PEQPHLPPEQ ETIPPSPIQN ALPQPQYPTV  600
CHVHLPAQQP PDNQAHFPHP PAQHPQKSYG KPSPQTEIHS SYPLEHVAEH  650
TGKKPAEYAH TKEQTYPCYS GASGLHPKNL LPKFPSDQSS KSTETPSEQV  700
LQEDFASANA GSLQSLPGTV VPVRIQTHVP SYGSVHYTSI SQILGQNSPA  750
IVICKVDERH TQRTLVTNAA KQGIGFNIAQ VLGQHAGLEK YPINKAPQTL  800
PLGLESSIPL CLPSTSDSVA TLGGSKRHLS PASSLELPHE TKQQKRVKEE  850
KHYGQIVEEL SAVELTNSDI KKDLSRPQKP QLVRQGCASE PRDGLQSGSS  900
SPSSLSPSSS QQYPSVSPSS REPPPPSKEH LSGSRAPLPG QKSSGPSESK  950
ESSDELDIDE TASCMSHSPQ SSSLPAGDGQ LHEEGKGHKR PVGHLVRHAS 1000
APSGNVADST LLLTDHADFQ QILQFPSLRT TTTVSHCFLN YTKPNYVQQA 1050
TFESSVYASV CISSCHPHPS GLNTKTTLAL LASKQKITAE IYTLAAHHRP 1100
GTGKLTSSSA VKQFTQHKPD ASFLPGSKLE RKLVGNILKE RGRGDIHGDK 1150
DIGSEQTEPI RIKIFEGGYK SNEDYVYVRG RGRGKYICEE CGIRCKKPSH 1200
LKKHIRTHTD VRPYVCKLCN FAFETKGNLT KHMKSKAHHK KCLELGVSHT 1250
SVDDTETEEA ENLEDLHKAA EKHSHSSIST DHQFSDAERS DGEDGODNDD 1300
DDEDEDDFDD QGDLTPETRS RSTSPQPPRF SSLPVNVGAV PHGVPSDSSL 1350
GHSSLISYLV TLPSIRVTQL HTPSDSCEDT QSTEYQRLPQ SKSTDSEPDK 1400
DRLDIPSCHD EBCHLPSEPS SSPRDPSPSS HHSSPGYDSS PCRDNSPKRY 1450
LIPEGDLSPR RHLSPRRDLS PHRHLSPERE AALRREHSQR DVSPRRHLSP 1500
RRPVSPGKDI TARRDLSPRE ERRYHTTIRA PSPRRALYHN PPLSHGQYLQ 1550
AEPIVLGPPH LRRGLPQVPY FSLYGDQEGA YEHPGSSLPP EGPWDYVFSH 1600
LPLHSQQQVR APIPHVPVGG IQSVHSHPPA LSSLHPSPTL PLPHEGPEEK 1650
KGASGESPSK DPYVLSKQHE KRGPHALQSS GPPSTPSSPR LLHKQSTSED 1700
SLNATERERQE ENIQTCTKAI ASLRIATERA ALLGPDQPAR VQEPHQNPLG 1750
SAHVSIRHPS RPEPGQPCTS ATHPDLHDGE KDNPGTSQTP LAHSTFYSKS 1800
CVDQKQLDFH SSKELSSSTE ESKDPSSEKS QLH                    1833
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FIG. 2. **Amino acid sequence of HIV-EP2.** The nucleotide sequence was determined and is available from the GenBank data base. Only the 1,833 amino acids encoded by the large open reading frame of HIV-EP2 cDNA are shown. Two metal-finger structures are delineated by *heavy underlines*. The potential nuclear localization signal indicated by a *double line* is followed by the Ser/Thr-rich domain, which is shown with a *thin underline*. The cluster of acidic amino acids is indicated by *broken underlining*.

binding protein 2 (HIV-EP2) encoded by the full length mRNA is 202,100.

Comparison of the deduced amino acid sequence of HIV-EP2 with that of PRDII-BF1/MBP-1/HIV-EP1 showed that the three regions I, II, and III are highly homologous between the two proteins (Fig. 3). Region I involves the putative nuclear localization sequence, Pro-Lys-Lys-Lys-Arg-Leu-Arg, that is very similar to that in SV40 large T antigen, followed by the Ser/Thr-rich region. Region II corresponds to the DNA-binding domain consisting of two metal-finger structures. The structure of the first metal finger is completely conserved, but 3 amino acids in the second metal finger are different between the two proteins. Region III, which is immediately downstream from the metal-finger domain, contains the acidic amino acid-rich domain. In this region of HIV-EP1 and HIV-EP2, 15, and 16 residues are acidic amino acids, respectively.

Specificity of Binding to DNA—For the analysis of DNA-binding activity of HIV-EP2, the *lac*Z-HIV-EP2 fusion gene was inserted into a T7 expression vector. The *lac*Z-HIV-EP2 fusion protein encoded by this gene contained the first 1,008 amino acid residues of β-galactosidase fused to the 286 amino acid residues derived from the HIV-EP2 cDNA that contained the metal-finger domain of HIV-EP2. Fig. 4*A* shows the proteins in crude bacterial extracts. Large amounts of *lac*Z-HIV-EP2 protein were produced and accumulated within the cells. The identity of this protein was checked by Western blot analysis using the antibody against β-galactosidase and analysis of the amino acid composition (data not shown). This fusion protein was purified using the sequence-specific affinity column in which the HIV-enhancer sequence was used as a ligand. To examine whether HIV-EP2 binds directly to the HIV-1 enhancer, we used the purified *lac*Z-HIV-EP2 fusion protein for DNase I footprint experiments. The fusion protein gave two 10-nucleotide protected areas in the region of the HIV-1 enhancer in both strands (Fig. 4*B*). On the other hand, the *lac*Z-HIV-EP1 fusion protein gave two 7-nucleotide protections in the same DNA region (15). This difference may result from the DNA-binding property of two proteins or from the size of two fusion proteins.

MBP-1 was demonstrated to have the highest affinity for the MHC and β_2-microglobulin sites and approximately 5- to 10-fold lower affinity for the related κ-immunoglobulin and HIV-enhancer sites (17). To examine whether HIV-EP2 binds to these related κ sites with an affinity similar to MBP-1/HIV-EP1, gel mobility shift assays were done using the *in vitro* translated HIV-EP2. The 1,469-amino acid protein containing the HIV-EP2 metal-finger domain was synthesized by an *in vitro* translation system. This protein bound to the ^{32}P-labeled MHC-enhancer probe and generated protein-DNA complexes (Fig. 5, *lanes 1* and *10*). By using cold DNA competitors containing sites from the MHC, κ-immunoglobulin, and HIV enhancer, competition analysis was done. Addition of a 20-fold molar excess of the MHC competitor oligonucleotide completely abolished the complex formed with the MHC probe, but the κ-immunoglobulin and HIV-enhancer oligonucleotides competed for binding less well than the MHC oligonucleotide (Fig. 5, *lanes 3–9*). Thus, HIV-EP2 binds to the MHC site more tightly than to the HIV enhancer, indicating that the relative binding affinity of HIV-EP2 to the related κB sites is very similar to that of MBP-1/HIV-EP1.

Expression of HIV-EP2 mRNA—To study the expression of HIV-EP2 mRNA, poly(A)$^+$ RNAs were prepared from various cell lines and Northern blotting was done (Fig. 6*A*). After hybridization with the HIV-EP2 probe under stringent conditions, only a single RNA species was detected, with an estimated size of about 9.5 kilobases. The level of HIV-EP2 mRNA was variable in the cell types analyzed, and relatively high levels of HIV-EP2 mRNA were detected in a T cell lymphoma (Molt-4), and carcinoma from the thyroid (TC78), vulva (A431), and colon (Colo320DM). This cell-type specificity of HIV-EP2 expression is similar to that of HIV-EP1 (data not shown). Recently, expression of MBP-1 was demonstrated to be inducible by mitogen and phorbol ester treatment of Jurkat T cells (17). Therefore, we examined the effects of this treatment on HIV-EP2 expression. RNA isolated from Jurkat cells 6 h after phytohemagglutinin (PHA) and phorbol ester 4β-phorbol 12-myristate 13-acetate (PMA) co-stimulation was analyzed in parallel with RNA from unstimulated Jurkat cells. Hybridization with the λHIV-EP2–4 cDNA probe indicated a marked increase in levels of HIV-EP2 RNA following stimulation (Fig. 6*B*, *upper panel*). The level of β-actin mRNA was not markedly changed by PHA

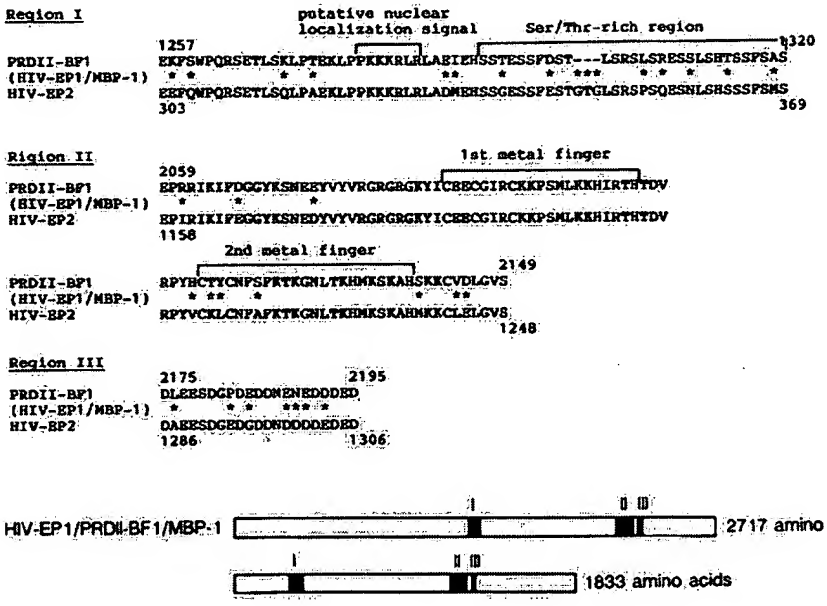

FIG. 3. **Homology between HIV-EP2 and PRDII-BF1/MBP-1/HIV-EP1.** The homology between HIV-EP2 and PRDII-BF1/MBP-1/HIV-EP1 in three regions are shown. *Asterisks* connect the different amino acid residues. *Dashes* indicate gaps that have been introduced for alignment. The amino acid numbers are indicated above or below the sequence. The positions of regions I, II, and III in the two proteins are indicated below.